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Circadian Rhythm of Calling Behavior in the Emei Music Frog (*Babina daunchina*) is Associated with Habitat Temperature and Relative Humidity

Jianguo CUI[1*#], Xiaoyan SONG[2#], Guangzhan FANG[1], Fei XU[1], Steven E. BRAUTH[3] and Yezhong TANG[1*]

[1] *Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu* 610041, *Sichuan, China*
[2] *Sichuan Institute of Animal Sciences, Chengdu* 610066, *Sichuan, China*
[3] *Department of Psychology, University of Maryland, College Park, MD* 20742, *USA*

Abstract Generally, the functions of vocalizations made by male anurans are to attract females or defend resources. Typically, males vocalize in choruses during one or more periods in a twenty-four-hour cycle, which varies, however, among species. Nevertheless, the causal factors influencing circadian variations of calling patterns in anuran species are not clear. In this study, male chorus vocalizations were monitored in the Emei music frog (*Babina daunchina*) for 17 consecutive days during the breeding season, while its habitat air temperature and relative humidity in the course of experiments were measured as well. The results revealed that the circadian calling patterns were characterized by two periods of peak vocalization, which were observed from 0500 h to 0700 h and from 1300 h to 2000 h, while the lowest activity period was found from 2100 h to 2200 h. Both calls/h and notes/h were positively correlated with air temperature and negatively with relative humidity. Overall, our data indicate that the Emei music frogs (*B. daunchina*) could regulate their vocal activities based on the changes of physical micro-environment (e. g., temperature or humidity) to maximize reproductive success.

Keywords rest-activity cycle, advertisement call, temperature, humidity, Emei music frog (*Babina daunchina*)

1. Introduction

Males of many anuran and insect species call in large choruses. Chorusing intensifies competition among males for attracting mates, acquiring and defending resources needed by females, or both. The choruses are thus focal points for sexual selection (Gerhardt and Huber, 2002; Wells, 2007). Choruses usually form in areas where the physical resources are required by females and offspring, and occur during seasonal and daily time periods that are favorable for courtship and reproduction (Wells, 1977; Thornhill and Alcock, 1983). Chorus vocalizations are thus influenced by circadian rhythms and are typically concentrated in one or more periods in a twenty-four-hour cycle (Greenfield, 1994; Runkle *et al*., 1994; Navas, 1996; Bridges and Dorcas, 2000).

In many anurans, male calling occurs for several hours at night and even for twenty-four hours under particularly favorable conditions (Drewry and Rand, 1983; Bridges and Dorcas, 2000). However, the factors that affect calling behavior remain unclear. For anurans, weather conditions such as air temperature are often associated with the timing and intensity of episodes of reproductive activity which in both males and females are subject to energetic constraints (Gerhardt and Huber, 2002). In addition, the availability of water for laying eggs is critical for anuran reproduction, and thus humidity has been postulated to be involved in the regulation of calling activity. In the present study, we investigated the circadian rhythms in male calling behaviors in *B. daunchina* (Frost, 2009), as well as the possible relationship of calling behaviors with air temperature and relative humidity.

Emei music frog (*B. daunchina*), with an adult body

# These authors contributed equally to this work.
* Corresponding authors: Dr. Jianguo CUI, from Chengdu Institute of Biology, Chinese Academy of Sciences (CIB), with his research focusing on behavioral ecology of amphibians, and Prof. Yezhong TANG, from CIB, with his research focusing on behavioral neuroscience of amphibians and reptiles.
E-mail: cuijg@cib.ac.cn; tangyz@cib.ac.cn
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length of 45-50 mm, is also called "fairy player" by the local residents in this region, but it was first designated as *Rana musica* by Chang and Hsu (1932). This species inhabits the ponds covered with weeds during the breeding season in southwestern China at altitudes ranging between 900 m and 1800 m where the climate is wet and temperate (Ye *et al.,* 1993). From May to August, the males build mud-based burrows in muddy areas under the shadow of weeds, and produce calls of varying lengths consisting of a series of "musical" notes from inside or outside the burrows (Cui *et al*., 2010; Chen *et al*., 2011). Considerable individual variation in the durations, numbers of notes and note modulation patterns have been found in this species, although the causes of this variability are not well understood (Chen *et al*., 2011). In the present study, we investigated the correlation of air temperature and humidity with the production of male calls. We hypothesized that these environmental variables are essential factors in call production as well as their reproductive success in the Emei music frog.

2. Materials and Methods

2.1 Study site and subjects The study site is located in the area of Mt. Emei (29.36° N and 103.22° E, elevation of 941 m above sea level), Sichuan, China. During June and July, 2010, experiments were conducted in a nearly round pond with a diameter of approximately 2 m, which is colonized by and provided a natural habitat for *B. daunchina*. The middle part of the pond was covered with water, while the surroundings were muddy and sheltered by sparse or dense weeds. Shrubbery and weeds flourished along the banks of the pond in June and July. Male frogs built nests in the muddy area covered with grasses. The nests were scattered between water and pond bank. There were about 20–25 male frogs in this pond at the time of the study.

2.2 Vocalization recordings The animal treatment procedures were approved by the Animal Care and Use Committee of the Chengdu Institute of Biology, Chinese Academy of Sciences. To investigate call production in *B. daunchina*, the chorus of male frogs in the pond was recorded continuously for twenty-four hours each day from 28 June to 14 July, 2010, using an Aigo R5518 recorder with internal microphone (Aigo Digital Technology Co. Ltd, Beijing) which was placed at the pond bank. Figure 1 depicts the examples of advertisement calls recorded from one male frog using a Sennheiser ME66 directional microphone (with K6 power module) connected to Marantz PMD 660 recorder (16 bit, 44.1 kHz) about 1 m from the subject.

2.3 Measurements of temperature and relative humidity Air temperature and relative humidity were sampled automatically every hour using a TP220USB temperature and relative humidity recorder (Beijing Anfu Electronic Technique Co., Beijing), which was placed at the pond bank. These data were then stored on the hard drive of a computer after all the experiments were completed.

2.4 Analysis and statistics The amplitude modulated waveforms (oscillograms) and sonograms of advertisement calls were analyzed using PRAAT software (Boersma & Weeninkk, Version 5.1.11, University of Amsterdam). The calls consisted of 3–8 notes which are short discrete sounds produced in succession. The notes and call renditions were enumerated manually using Adobe Audition 3.0 (California, USA) software, and the numbers of calls/h, notes/h and notes/call were enumerated.

Data were statistically analyzed using PASW Statistics 18.0 software (SPSS Inc., Chicago, IL, USA). Prior to the statistical analyses, all data were examined for assumptions of normality and homogeneity of variance, using Kolmogorov-Smirnov and Levene tests, respectively. One way repeated measures ANOVA was employed to evaluate the differences in calls/h, notes/h, notes/call, temperature and relative humidity in different time periods. The *Bonferroni* test was used for *post hoc* comparisons between different time points. Spearman's correlation analysis was used to detect the possible relationships among the variables. The data are expressed as mean ± SD, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Acoustic properties of male advertisement calls Consistent with previous studies (cf. Chen *et al*., 2011), most male advertisement calls were composed of 3-8 notes, the durations of which varied from 100 to 200 ms. In the frequency domain, the notes were composed of 1-4 obvious harmonics, the fundamental frequency of which varied from 455 Hz to 597 Hz (from the first to the sixth note; Figures 1 A, B).

3.2 Diurnal patterns of calling behavior, temperature and relative humidity Calling behavior, i. e., calls per h ($F_{23,299} = 6.647$, $P < 0.001$; Figure 2 A), notes per h ($F_{23,299} = 6.279$, $P < 0.001$; Figure 2 B) and notes per call ($F_{23,299} = 8.324$, $P < 0.001$; Figure 2 C) significantly varied across

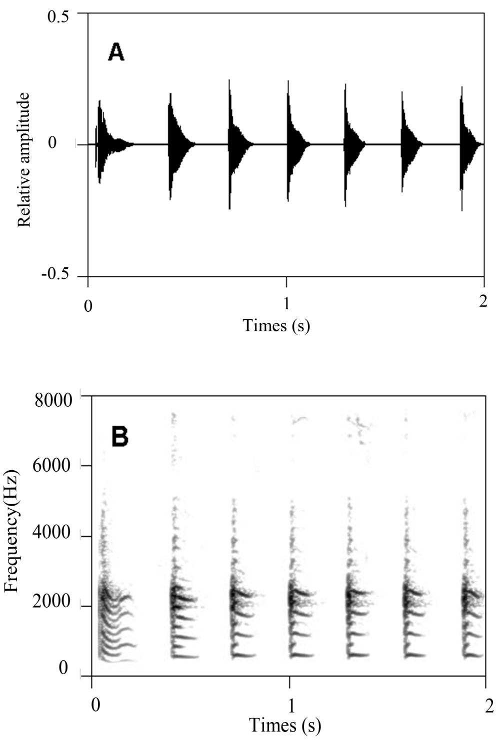

Figure 1 Amplitude-modulated waveform and spectrogram of a typical male advertisement call of *B. daunchina*. The FFT frame is 1024.

the 24 h cycle. Repeated measures analysis showed that there were two periods of peak calling activity, from 0500 h to 0700 h and from 1300 h to 2000 h, while the lowest activity period was found from 2100 h to 2200 h (Figures 2 A, B, C). Spearman's correlation analysis showed that the notes per call was positively correlated with calls per h (r = 0.802, P < 0.001, n = 384; Figure 3 A) (n = 24 h/d × 16 d = 384). Pond air temperature significantly varied during the day ($F_{23,368}$ = 26.997, P < 0.001; Figure 2 D), with a mean value of 21.5 ºC at 0100 h which decreased to 20.9 ºC at 0400 h, where it remained relatively stable until 0800 h before rapidly increasing from 21.8 ºC at 0900 h to a peak value of 27.8 ºC at 1600 h, and then it decreased gradually to 21.9 ºC at 2400 h (Figure 2 D). Relative humidity also varied significantly at the pond within 24 h ($F_{23,368}$ = 21.537, P < 0.001), although humidity varied inversely with temperature (Figure 2 D) (r = - 0.733, P < 0.001, n = 408; Figure 3 B). The lowest point of the relative humidity was 72.57 ± 6.04 % at 1600 h, while the highest was 94.15 ± 16.23 % at 0600 h (Figure 2 D).

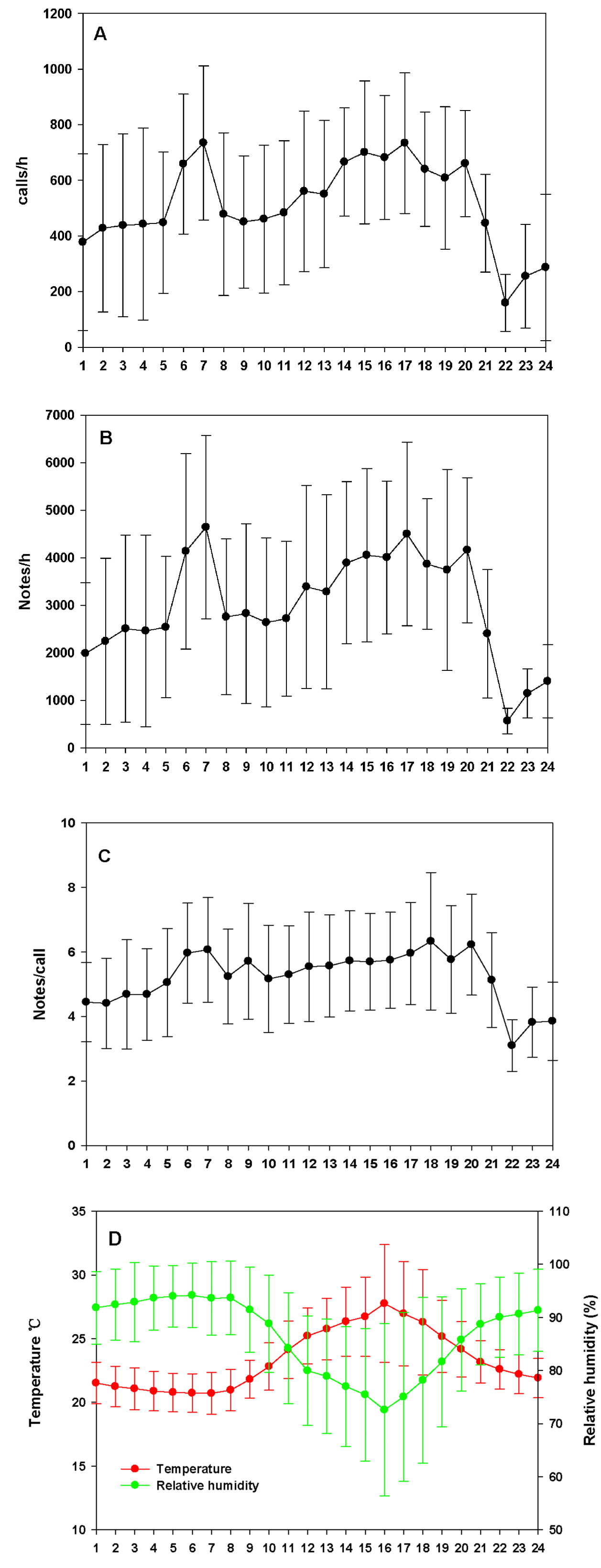

Figure 2 Graphs showing the daily variations in calls/h (A), notes/h (B), notes/call (C), and temperature and relative humidity (D) in a 24 - h period for *B. dauchina* (n = 17, mean ± SD).

3.3 Relationship among temperature, relative humidity and calling behavior Relationships between mean

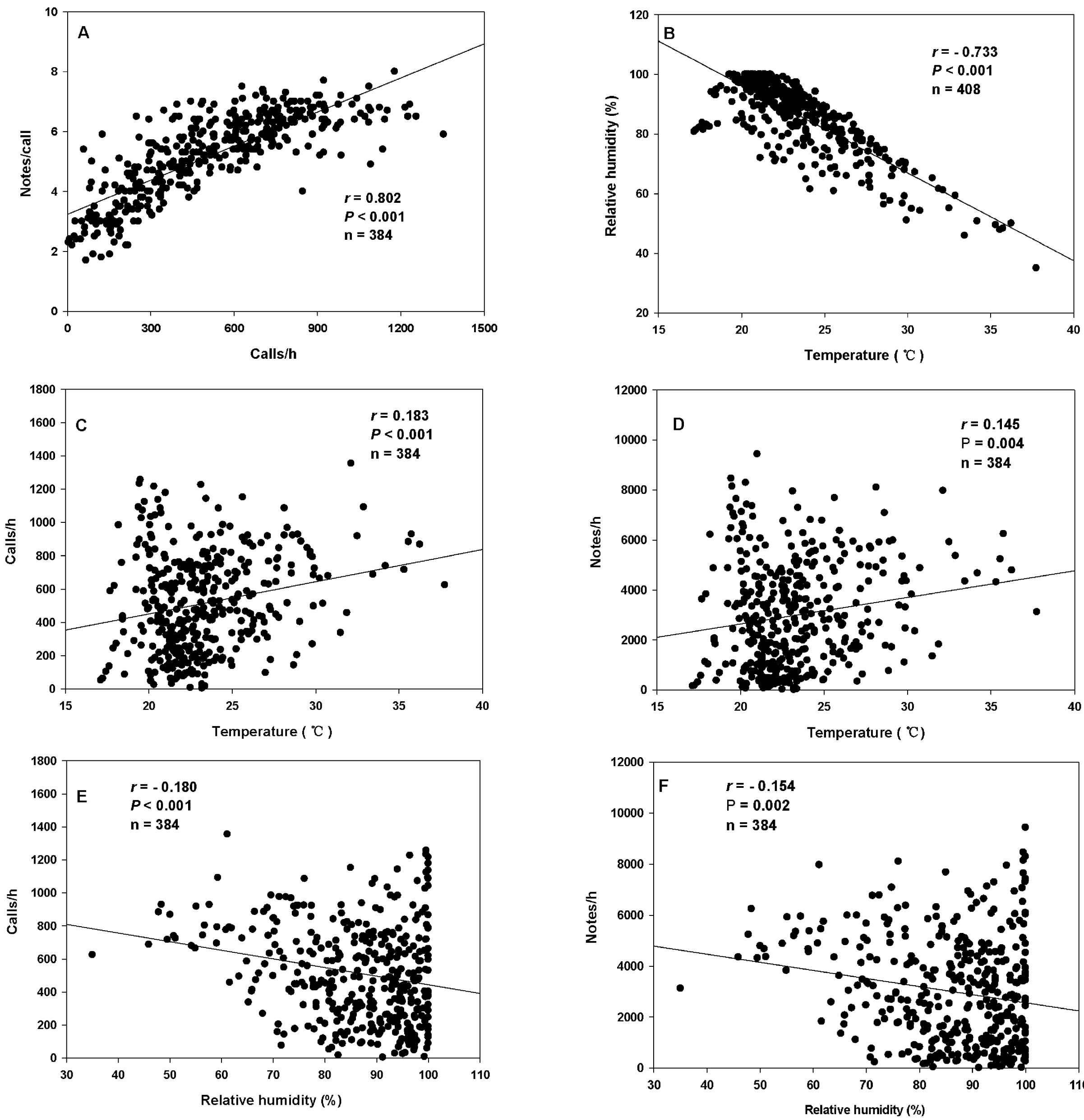

Figure 3 Graphs illustrating daily variations in notes/call and calls/h (A), relative humidity and temperature (B), calls/h and temperature (C), notes/h and temperature (D), calls/h and relative humidity (E), and notes/h and relative humidity (F) for *B. daunchina.*

hourly values of air temperature and relative humidity with the acoustic properties of advertisement calls produced during these time periods were examined using Spearman's correlation analysis from 28 June to 14 July, 2010. The results showed that both calls/h (r = 0.183, P < 0.001, n = 384; Figure 3 C) and notes/h (r = 0.145, P = 0.004, n = 384; Figure 3 D) (n = 24 h/d × 16 d = 384) were positively correlated with air temperature, while negatively with relative humidity (r = - 0.180, P < 0.001, n = 384; Figure 3 E, and r = - 0.154, P = 0.002, n = 384; Figure 3 F). Given that the second peak of calling activity was well consistent with the changes in the temperature and humidity, we extracted the data from 1200 h to 2400 h and analyzed the data separately. The results showed that the above correlations remained while the r values increased. Both calls/h (r = 0.437, P < 0.001) and notes/h (r = 0.398, P < 0.001) were positively correlated with air temperature, while negatively correlated with relative humidity (r = - 0.392, P < 0.001; and r = - 0.361, P < 0.001) (n = 12 h/d × 16 d = 192).

4. Discussion

The results of the present study show that male *B. daunchina* calling behavior exhibits a distinctive circadian pattern during the breeding season. Habitat air temperature and relative humidity likely affect these diurnal rhythms insofar as daily variations in temperature and humidity are significantly correlated with the changes in call parameters.

Few studies have examined the daily variations in anuran calling activity although the available evidence indicates that the patterns of calling activity across the 24 - hour cycle differ substantially among species. Bevier (1997) found considerable variation in tropical anurans in Panama, and Runckle *et al*. (1994) found that the calling of *Hyla versicolor* peaked in early evening. Given (1987) reported that the calling activity of *Rana virgatipes* peaked around midnight, and Shimoyama (1989) reported that *R. porosa* called more frequently after midnight than before midnight. Interestingly, the pattern of calling activity of *B. daunchina* differs from those of the species described above, insofar as calls/h, notes/h and notes/call peaked twice during the day from 0500 h to 0700 h and from 1300 h to 2000 h, while the lowest activity period occurred from 2100 h to 2200 h. Furthermore, in the Emei music frog during the breeding season, the vocal activity in chorus continued for 24 h in most of the study days.

Although previous studies have shown that air temperature and relative humidity are important factors affecting reproductive activity and calling behavior in anurans and insects (Navas, 1996; Wong *et al*., 2004; Saenz *et al*., 2006), these studies were not based on 24 - h continuous recording. In the present study 24 - h continuous recording revealed that air temperature peaked at 1300 h to 1400 h when relative humidity reached its lowest point. Notably this time period corresponds closely to one of the two peaks of the calling activity of *B. daunchina*. Moreover, correlation analysis confirms that male *B. daunchina* calling activity is positively correlated with air temperate and negatively with relative humidity. These data suggest that relatively higher temperatures and lower humidity facilitate the chorus in this species.

In some temperate species, signaling ceases as temperature falls below some critical level or as energetic reserves required for calling become low. Air temperature affects not only the duration of calling, but also male and female locomotor activities in insects that fly toward calling males (Walker, 1983). In tropical environments, temperature can become too high to sustain continuous signaling during the day, so that signaling commences in the evening when temperature falls (Gerhardt and Huber, 2002). While energy limitations almost certainly determine the end of nightly signaling in some species (Gerhardt and Huber, 2002), the end of chorusing in the barking treefrogs (*Hyla gratiosa*) is correlated with the cessation of female arrival (Murphy, 1999).

In addition to air temperature, amphibians also respond behaviorally to fluctuations in water availability (Dole and Durant, 1974; Pough *et al*., 1983; Cree, 1989) and rainfall events (Saenz *et al*., 2006). Navas (1996) reported that occasional diurnal activity of *Eleutherodactylus bogotensis* at 3500 m likely reflects a release from hydric constraint, because at that site the soil is usually saturated with water. Although only a few species have been studied, *Eleutherodactylus* appears to be sensitive to water loss (Pough *et al*., 1983) and tends to be more active when the foliage is wet (Woolbright, 1985). Thus, higher relative humidity would be expected to correlate with higher vocal activity. Nevertheless, higher relative humidity apparently seems to inhibit the vocal activity of *B. dauchina.* This might be explained by the fact that during the breeding season of 2010, relative humidity was higher than the optimal level for calling. As can be seen in Figure 3, the relative humidity was quite high in the days when the frogs were recorded, ranging from 50% to as high as 100%. Additional work is needed to determine if these conditions occur on a regular basis during the breeding season.

Although peak male calling in this study occurred close to the point of maximum air temperature, the correlation coefficients (r values) for both air temperature and relative humidity with calling activity, though statistically significant ($P < 0.001$), were relatively small (absolute value < 0.2). However, we extracted and analyzed the data from 1200 h to 2400 h, and the results showed that the above correlations remained while the r values increased. These results suggested that the peak of the call activity in morning might be associated with the photoperiod, while the second peak in afternoon would be with temperature and relative humidity. In conclusion, the circadian rhythm of calling behavior in the Emei music frog is associated with habitat air temperature and relative humidity, and other factors are also likely important such as endogenous rhythm, photoperiod (Jaeger, 1981) and social factors (Brooke *et al*., 2000; Oseen and Wassersug, 2002) which deserve further study.

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